Changes in Human Endothelial Cell Energy Metabolic Capacities during *in vitro* Cultivation. The Role of “Aerobic Glycolysis” and Proliferation

Kirsten Peters^{1*}, Günter Kamp^{2*}, Ansgar Berz², Ronald E. Unger³, Susanne Barth³, Achim Salamon¹, Joachim Rychly⁴ and C. James Kirkpatrick³

¹Junior Research Group, Department of Cell Biology, Medical Faculty, University of Rostock, Rostock, ²Institute of Zoology, Johannes Gutenberg University, Mainz, ³Institute of Pathology, Johannes Gutenberg University, Mainz, ⁴Department of Cell Biology, Medical Faculty, University of Rostock, Rostock, *these authors contributed equally to this work

Key Words

Endothelial cells • *In vitro* • Energy metabolism • Aerobic glycolysis • Proliferation

Abstract

Background: In this study the influence of cultivation and proliferation on energy metabolic characteristics of human umbilical vein endothelial cells (HUVEC) has been examined. The energy metabolic capacities of human endothelial cells freshly isolated from the umbilical vein were compared with those after cultivation for three passages and as subconfluent and confluent cultures. **Methods:** Expression of cell type-specific differentiation markers and proliferative activity were studied in dependency on cultivation characteristics. Furthermore, the energy metabolic characteristics of HUVEC were analyzed by measurement of the maximum catalytic activities of marker enzymes of various metabolic pathways. **Results:** Examination of a typical marker of proliferation, Ki67, confirmed that HUVEC changed in culture from a non-proliferative to a proliferative state. Compared to other cell types, the enzyme pattern of HUVEC showed a high glycolytic and a high NADPH regenerating capacity. These capacities increased by cultivation nearly to

the same degree as marker enzymes of other metabolic pathways (e.g. citric acid cycle). **Conclusion:** Our data support the theory that metabolism of EC is primarily by “aerobic glycolysis”, i.e. the conversion of glucose to lactate in the presence of oxygen. These characteristics were independent of whether the cells are freshly isolated/non-proliferating or cell culture-adapted/proliferating.

Copyright © 2009 S. Karger AG, Basel

Introduction

Vascular endothelial cells (EC) line blood vessels in every organ and tissue in the body and are estimated to consist of approx. 1 to 6 x 10¹³ cells in adult humans which accounts for a significant share (about 1%) of the total body weight [1]. EC come into contact with all blood components such as nutrient substances including oxygen, biologically active molecules such as hormones, blood cells and drugs. It is well documented that EC play a crucial role in the regulation of blood flow and pressure [2], as well as blood coagulation [3]. Furthermore, EC are involved in inflammation [4], blood vessel formation [5] and are responsible for the bi-directional transmission

of biochemical and physical information between the blood and other vascular cells (e.g. smooth muscle cell population) and peri-vascular tissues, respectively. With these features, EC take part in the development of multiple diseases like atherosclerosis, thrombosis, sepsis, and tumor invasion and growth.

Since Jaffe et al. developed a method for the enzyme-based isolation of EC from human umbilical vein in the early 70's [6] the techniques of EC isolation and cultivation have increased tremendously. Nowadays, the cultivation of many different EC types from varying organ systems of different species is established (e.g. [7-9]). These endothelial cultures are used for a wide spectrum of *in vitro* testing often allowing inferences on the *in vivo* situation [10-12]. For example, vascular EC *in vitro* express specific adhesion molecules when activated by inflammatory mediators similar to the *in vivo* situation [13, 14]. Furthermore EC *in vitro* can be stimulated to angiogenic differentiation. Thus, human EC can be used for *in vitro* assays which permit quantitative analysis of anti- and pro-angiogenic potential of drugs [15, 16].

There is, however, evidence for a number of apparent discrepancies between EC *in vivo* and *in vitro*. For example, the expression of CD34 (transmembrane molecule on human hematopoietic progenitor cells and on many EC types) is reduced in EC of the human umbilical vein (HUVEC) with cultivation time [17, 18]. CD34 expression appears to be regulated by changes of the extracellular environment (growth factors, shear stress, extracellular matrix, further cell types/cell contact etc.) [19]. Furthermore, Kanda et al. revealed a decrease in the expression of the glucose transporter type 1 (GLUT1) in cultivated bovine peripheral nerve microvascular EC [20]. These cultivation-induced changes lead to the question of whether the energy metabolism in cultured EC is different from that *in vivo*.

Studies on EC of different origins have revealed that energy metabolism of EC in culture is based on glycolysis for ATP-production. Coronary EC from rats degrade 99% of glucose to lactate even under aerobic conditions but they are able to oxidize glutamine, lactate or fatty acids as well [21, 22]. Furthermore, Loike et al. discovered that HUVEC contain phosphocreatine and express creatine kinase (CK) [23] while Culic et al. (1997) did not find phosphocreatine in porcine EC [24]. Due to the low CK activity of EC *in vitro* a minor role of phosphocreatine to buffer cellular ATP level was suggested [25]. The pentose-phosphate pathway, which is important for anabolic processes and the defense against oxidative stress, is also present in EC as indicated by activities of

glucose 6-phosphate dehydrogenase (G6PDH) [26]. Since these data are gained from EC *in vitro*, there are open questions with respect to possible cell culture-related adaptation effects.

Due to the widespread distribution of EC within different tissues, energy metabolism *in vivo* can not be investigated in a quantitative manner as can be done with other organs (e. g. muscle, liver or brain), in which the tissue-specific cell-type accounts for the largest cell population within the tissue. Hence, most data concerning the energy metabolism of EC are derived from EC after proliferation in culture. How far cell cultivation and thus related proliferation affects the metabolic pattern of EC is still unknown. Renal epithelial cells, for example, show dramatic alterations of metabolic rates and levels of enzyme activities by changes in nutrient supply and oxygenation *in vitro* [27].

EC *in vivo* are evaluated as proliferatively quiescent in adults (if not activated by regeneration processes), whereas EC *in vitro* possess a proliferation-activated phenotype [1]. Dobrina and Rossi investigated freshly isolated microvascular EC of calf penis and found high rate of glycolysis compared to that of respiration and a high potential of pentosephosphate pathway as described in EC in confluent cultures [28]. In this paper we present a human EC-based comparative study of energy metabolism of freshly isolated EC with cultivation-adapted, proliferating and proliferation-reduced EC.

We have established an isolation protocol for EC from human umbilical vein that led to a sufficient number of cells for subsequent enzyme analysis. This cell population was called P₀ since no cell passaging took place. These P₀-HUVEC cultures were almost free of contaminating cells (blood cells, perivascular cells) and thus represent a suitable basis for the determination of maximum activities of marker enzymes representing different pathways of energy metabolism *in vivo*. The P₀-data were compared with those from HUVEC kept in culture for approximately two weeks (including three passages) reaching a subconfluent (sP₃) or confluent state (cP₃). In addition the enzyme activities were compared with data of other cell types in order to highlight the specific characteristics of energy metabolism in HUVEC.

Materials and Methods

Materials

Cell culture plastic was obtained from Becton Dickinson and Greiner if not otherwise indicated.

Isolation and culture of EC

EC from the human umbilical vein (HUVEC) were isolated as described by Jaffe et al. [6]. Since cultures were classified into P₀ and P₃, two different cell isolation procedures were used. For P₀ cultures, the cells were seeded in fetal calf serum (FCS)-containing M199 (composition see below) and kept 4 h in the incubator for cell attachment. After 4 h cells were fixed for cell characterisation (see 'Immunofluorescence') or cell lysates were made for enzyme activity assays (see below). In contrast, the P₃ cultures were cultivated until confluency was reached (within 3–4 d). Cell cultivation was performed in M199 (Sigma-Aldrich) containing 20% FCS (Invitrogen), 2 mM GlutamaxTM-I (Invitrogen), Penicillin/Streptomycin (100 U/ml and 100 µg/ml, Invitrogen), sodium heparin (25 µg/ml, Sigma-Aldrich), and endothelial growth factor supplement (25 µg/ml, Becton Dickinson) in a humidified atmosphere (37°C, 5% CO₂). The cells were cultured on tissue culture plastic pre-coated with gelatine (0.2%, Sigma-Aldrich). The culture medium was changed every two or three days. The cells were detached using trypsin-EDTA (Invitrogen).

Immunofluorescence

Tissues: Umbilical cords were cleaned from attached blood and the vein was washed three times with warm phosphate-buffered saline solution (PBS, Ca²⁺/Mg²⁺-free, 37°C, Cambrex) so that blood cells were largely removed. Afterwards the vein was filled with 4% formaldehyde (PBS-buffered, freshly prepared, Sigma-Aldrich) and the cord was totally immersed in formaldehyde. After the fixation (4 h, room temperature) the cord was cut in 0.5 cm slices and the slices were embedded in paraffin according to standard protocols. Paraffin sections were mounted on coated slides (SuperFrost Plus, Menzel), dewaxed, and rehydrated through alcohol gradients. Antigens were unmasked by microwave treatment of the slides (5 min, three times, citrate buffer pH 6.0, 700 W). Staining for von Willebrand Factor (vWF) was with a polyclonal rabbit antibody (Dako); staining for CD31 (platelet endothelial cell adhesion molecule-1/PECAM-1) and CD34 was with monoclonal mouse antibodies (CD31: clone JC70A, Dako; CD34: clone QbEnd/10, Dianova). The secondary, fluorochrome-coupled antibody was a goat-anti-rabbit or a goat-anti-mouse AlexaFluor 488, respectively (Invitrogen). The secondary antibody was used without the presence of a primary antibody as negative control. The slides were covered with GelMount (Biomedica Corp/Natutec).

Cells: Freshly isolated human umbilical vein cells or HUVEC in P₃ were seeded onto fibronectin-coated glass chamber-slides (Nunc). After 4 h (for freshly isolated cells) or after 3 passages (for cell culture-adapted HUVEC), respectively, cells were fixed with buffered 3.7% paraformaldehyde (15 min, room temperature, Sigma-Aldrich). Staining for vimentin, smooth muscle actin (SM-actin), CD14, CD31, CD34 and Ki67 was performed with monoclonal mouse antibodies (vimentin: clone VIM 3B4 - Progen; SM-actin: clone ASM-1 - Progen; CD14: clone 7 - Novo Castra/Loxo; CD31: clone HC1/6 - Cymbus Biotechnology; CD34 clone QBEnd/10 - Dianova, Ki67: clone MIB-1). Staining for vWF was performed with a polyclonal rabbit antibody (Dako). AlexaFluor 488-secondary antibodies were used for the final antigen detection (AlexaFluor 488 goat anti-mouse,

donkey anti-rabbit, Invitrogen). Nuclear staining was with Hoechst 33342. Fluorescence labeled cells were covered with GelMount (Biomedica Corp/Natutec).

Enzyme activity assays

HUVEC were enzymatically detached by discharging culture medium, washed with PBS and rinsed with trypsin/EDTA solution (0.25% trypsin/1 mM EDTA, Invitrogen) for a few seconds, the trypsin/EDTA solution was then removed and the dishes incubated for about 2 min until the HUVEC were detached from the surface. The enzymatic reaction was stopped by the addition of FCS-containing medium. Afterwards the cell suspension was centrifuged with 300 x g at 4°C for 5 min; the resulting supernatant was removed and the HUVEC pellet was shock-frosted in liquid nitrogen and afterwards stored at -20°C.

All chemicals addressed in the following section were purchased from Sigma-Aldrich (chemicals) or Roche (enzymes) if not otherwise indicated. For enzyme assays the frozen cell pellets were resuspended in sodium phosphate buffer (100 mMol/l; pH 7.6) containing 1 mMol/l EDTA, 1 mMol/l dithiothreitol and homogenized with sonifier (3 x 10 s, at 4°C). Enzyme activities were performed with aliquots of HUVEC homogenates. For comparison, frog gastrocnemius muscles, mouse hearts and flight muscles of the hawk moth *Manduca sexta* were homogenized in the same solution as HUVEC, centrifuged at 10,000 x g (10 min, 4°C) and the supernatant were used for assays. The assays were performed spectrophotometrically at 340 nm (25°C) using NAD(P)H + H⁺ as indicator. The activities are given as international units (1U = 1 µmol substrate transformed per min at 25°C). The enzyme assays are described in Bergmeyer (1983) and were modified as described [29]:

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12): 50 mmol L⁻¹ triethanolamine buffer (TRAP), 4 mmol L⁻¹ MgCl₂, 2 mmol L⁻¹ MgATP, 1 mmol L⁻¹ EDTA, 2.4 mmol L⁻¹ glutathione, 7 mmol L⁻¹ phosphoglycerate, 0.2 mmol L⁻¹ NADH + H⁺, 10 U mL⁻¹ phosphoglycerate kinase (EC 2.7.2.3); pH 7.6.

6-phosphofructokinase (PFK; EC 2.7.1.11): 50 mmol L⁻¹ triethanolamine buffer (TRAP), 50 mmol L⁻¹ KCl, 2 mmol L⁻¹ MgCl₂, 10 mmol L⁻¹ NaP_i, 1 mmol L⁻¹ AMP, 7 mmol L⁻¹ Glucose 6-phosphate, 2 mmol L⁻¹ fructose 6-phosphate (F6P), 0.2 mmol L⁻¹ NADH + H⁺, 2 mmol L⁻¹ MgATP, 1.8 U mL⁻¹ aldolase (EC 4.1.2.13), 13.6 U mL⁻¹ glycerol 3-phosphate dehydrogenase (GDH, EC 1.1.1.8), 20 U mL⁻¹ triosephosphate isomerase (TIM, EC 5.3.1.1), 2.8 U mL⁻¹ phosphoglucose isomerase (PGI, EC 5.3.1.9); pH 7.6. The auxiliary enzymes were extensively dialysed against 250 mmol L⁻¹ TRAP (pH 7.6) to remove (NH₄)₂SO₄.

Lactate dehydrogenase (LDH; EC 1.1.1.27): 50 mmol L⁻¹ triethanolamine buffer (TRAP), 5 mmol L⁻¹ EDTA, 2 mmol L⁻¹ pyruvate, 0.2 mmol L⁻¹ NADH + H⁺; pH 7.6.

NAD⁺-dependent isocitrate dehydrogenase (NAD-ICDH; EC 1.1.1.41): 80 mmol L⁻¹ PIPES buffer, 8 mmol L⁻¹ MgCl₂, 2 mmol L⁻¹ ADP, 2 mmol L⁻¹ NAD⁺, 15 mmol L⁻¹ isocitrate; pH 8.0.

NADP⁺-dependent isocitrate dehydrogenase (NADP-ICDH; EC 1.1.1.42): 80 mmol L⁻¹ imidazole/HCl buffer, 4 mmol L⁻¹ MgCl₂, 0.5 mmol L⁻¹ NADP⁺, 0.15 mmol L⁻¹ isocitrate; pH 8.0.

NAD(P)H oxidized-glutathione oxidoreductase (GSSG-red; EC 1.6.4.2): 88 mmol L⁻¹ Tris (hydroxymethyl)-aminomethan-

	GAPDH	PFK	LDH	NADP-ICDH	HOADH	CK	G6PDH
P ₀ (n=7)	270 ± 36	17 ± 3	208 ± 41	5.7 ± 0.8	1.4 ± 0.5	11.3 ± 0.4	9.2 ± 0.3
sP ₃ (n=4)	835 ± 169*	69 ± 51	333 ± 111	21.0 ± 8.6*	5.5 ± 2.0*	56 ± 30*	47 ± 17*
cP ₃ (n=7)	764 ± 218	64 ± 42	297 ± 33	25.8 ± 9.7	8.1 ± 3.0	51 ± 24	45 ± 17
sP ₃ /P ₀	3.1	4.1	1.6	3.7	3.9	5.0	5.1
cP ₃ /sP ₃	0.9	0.9	0.9	1.2	1.5	0.9	1.0

Table 1. Activities of energy metabolism-associated enzymes in HUVEC of P₀ and P₃ (subconfluent and confluent) and ratios of sP₃/P₀ (cell culture adapted and proliferating vs. non-cultivated and non-proliferating HUVEC) and cP₃/sP₃ (confluent and slightly proliferating vs. subconfluent and highly proliferating) respectively. Results are presented as means ± s.e.m. for at least four separate batches of cells (n). The maximum activities are given in μmol product formed per min and g protein at 25°C (acronyms: GAPDH/glyceraldehyde 3-phosphate dehydrogenase, PFK/6-phosphofructokinase, LDH/lactate dehydrogenase, NADP-ICDH/NADP⁺-dependent isocitrate dehydrogenase, HOADH/β-hydroxyacyl-CoA dehydrogenase, CK/creatinekinase, G6PDH/Glucose 6-phosphate dehydrogenase. *) data of subconfluent P₃ (sP₃) were significantly different from P₀ (P < 0.01). The data of confluent P₃ (cP₃) did not significantly differ from those of sP₃ at the same level.

HCl, 0.9 mmol L⁻¹ EDTA, 4.6 mmol L⁻¹ GSSG, 0.16 mmol L⁻¹ NADPH + H⁺; pH 8.0.

β-Hydroxyacyl-CoA dehydrogenase (HOADH; EC 1.1.1.35): 100 mmol L⁻¹ triethanolamine buffer (TRAP), 5 mmol L⁻¹ EDTA, 0.2 mmol L⁻¹ NADH + H⁺; pH 7.6.

Creatinekinase (CK; EC 2.7.3.2): 100 mmol L⁻¹ imidazole/HCl buffer, 10 mmol L⁻¹ Mg acetate, 2 mmol L⁻¹ EDTA, 2 mmol L⁻¹ ADP, 10 μmol L⁻¹ diadenosinepentaphosphate, 30 mmol L⁻¹ phosphocreatine, 2 mmol L⁻¹ NADP⁺, 2 U mL⁻¹ glucose 6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49), 3 U mL⁻¹ hexokinase (HK, EC 2.7.1.1); pH 7.6.

Glucose 6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49): 50 mmol L⁻¹ triethanolamine buffer (TRAP), 5 mmol L⁻¹ EDTA, 0.4 mmol L⁻¹ NADP⁺, 2 mmol L⁻¹ glucose 6-phosphate; pH 7.6.

Fructose-1,6-bisphosphatase (FBPase, EC 3.1.3.11): 100 mmol L⁻¹ Tris (hydroxymethyl)-aminomethan-HCl, 5 mmol L⁻¹ EDTA, 80 mmol L⁻¹ MgCl₂, 0.4 mmol L⁻¹ fructose 1,6-bisphosphate, 0.4 mmol L⁻¹ NADP⁺, 0.7 U mL⁻¹ G6PDH (EC 1.1.1.49), 2.8 U mL⁻¹ phosphoglucose isomerase (PGI, EC 5.3.1.9); pH 7.5.

Protein quantification

The protein quantification was performed by the BCA Protein Assay Kit (Pierce) according to the manufacturer's instructions.

Number of determinations and statistical analysis

Numbers of determinations are mentioned in the Figure and Table legends. Minimal number was n=3, highest was n=9. Calculations and statistical analysis were carried out with Excel 2003. The homogeneity of variance of enzyme activity data was analyzed with the F-test. Differences between the mean were analyzed by t-test (P < 0.01 as indicated in Table 1).

Results

EC-specific markers in freshly isolated and culture-adapted HUVEC

Human EC from umbilical vein (HUVEC) were isolated within 4 h (P₀) and kept in culture for approx. 2 weeks with three passages (P₃). In order to verify that the freshly isolated HUVEC (P₀) were free of contaminating cells of other types, cell type-specific immunofluorescent staining reactions were performed. HUVEC *in situ* exhibit strong expression patterns for CD34, von Willebrand Factor (vWF) and CD31 (Fig. 1a-c) and in agreement with the *in vivo* situation the freshly isolated cell population was positive for these markers (Fig. 1d: CD34; Fig. 1e: vWF; Fig. 1f: CD31). The staining pattern was typical for endothelium. The vWF was stored in Weibel-Palade-bodies so that the staining appeared punctated. CD31 was distributed within the intercellular contacts. Furthermore, cells were negative for smooth muscle cell (SMC)-specific actin (SM-actin) and the cell surface marker CD14, specific for monocytes/macrophages and neutrophils (data not shown). On the basis of this staining pattern we concluded that the cell population was of endothelial origin. Thus, a noticeable contamination of the freshly isolated EC with blood cells, smooth muscle cells and fibroblasts could be excluded. After about two weeks in culture (from P₀ to P₃) the protein expression of CD34 was reduced. Whereas cells in P₀ showed CD34 protein expression, only a few CD34-

Fig. 1. Immunofluorescence staining for CD34, vWF and CD31 of the HUVEC layer in human umbilical vein cross sections (a,b,c), of freshly isolated cells (P_0 , 4 h after isolation; d,e,f) and of confluent HUVEC in P_3 (cP_3 ; g,h,i). a,d,g) CD34 (green), b,e,h) vWF (green), c,f,i) CD31 (green; nuclear staining in blue).

Fig. 2. Proliferative activity of freshly isolated HUVEC (P_0) and of cell culture adapted cells in P_3 ; a) immunofluorescence staining for Ki67 protein in P_0 -cultures 4 h after isolation and b) in sP_3 -cultures (Ki67 staining in green, nuclear staining in blue); c) phase contrast microscopic depiction of the subconfluent state of sP_3 -cultures (2 days after seeding in P_3) and d) of the confluent state in cP_3 -cultures (4 days after seeding in P_3), e) relative DNA amount in P_3 -cultures (determined by crystal violet staining 2, 4, and 5 days after seeding, sP_3 : 2 days after seeding, cP_3 : 5 days after seeding, $n=9$, *significantly different from sP_3 -cultures which are set as 100%, $p>0.001$); f) Ki67 protein expression/DNA amount in P_3 -cultures 2, 4 and 5 days after seeding ($n=6$, *significantly different from sP_3 -cultures which are set as 100%, $p<0.05$).

positive HUVEC were detectable in P_3 (Fig. 1g). Interestingly, the intensity of CD34 protein expression in the CD34-positive subpopulation appeared as intense as in P_0 (Fig. 1d). The amount and distribution of vWF (Fig. 1h) and CD31 (Fig. 1i) were similar in P_0 and P_3 , vWF was stored in Weibel-Palade-bodies, and CD31 was distinctly detectable within the interendothelial cell contacts.

Cultivation length and cell density-dependent changes of EC- and proliferation-specific markers in HUVEC

The proliferation marker Ki67, strictly associated with cell proliferation and thus present during all active phases of the cell cycle, was almost completely lacking in P_0 HUVEC (Fig. 2a). Thus, HUVEC freshly isolated from umbilical cords did not exhibit a proliferative activity. In contrast to P_0 cultures, subconfluent cultures of HUVEC in P_3 (sP_3 , 2 days after seeding, Fig. 2c) showed

a distinct Ki67 protein expression (Fig. 2b). Approximately 20% of the cells were Ki67 positive (estimated by counting Ki67-positive cells and nuclei). 4 days after seeding in P_3 the cultures reached confluency (Fig. 2d). At that time point the cell number was 2.4-fold higher compared

	HOADH/PFK	LDH/ICDH	LDH/PFK	G6PDH/PFK	GSSG-red./PFK
HUVEC sP ₃ (n=4)	0.12 ± 0.04	28 ± 12	12 ± 4	0.68 ± 0.12	0.32 ± 0.09
white muscle, frog gastrocnemius (n=4)	0.07 ± 0.02	130 ± 22	24 ± 4	< 0.001	0.02 ± 0.01
red muscle, mouse heart (n=3)	2.5 ± 0.6	30 ± 5	23 ± 4	0.09 ± 0.03	0.07 ± 0.03
flight muscle, <i>M. sexta</i> (n=4)	21.1 ± 8.3	0.02 ± 0.01	0.05 ± 0.03	< 0.001	< 0.001
fibroblasts (MRC-5) ¹⁾		4.3	4.9	0.4	
epithelial larynx carcinoma cells ¹⁾		111	88.6	0.4	
tumorigenic cells of rat heart (RCC-It) ¹⁾		135	197.5	0.2	

Table 2. Comparison of enzyme patterns of HUVEC (sP₃) with those of other tissues and cell types. Marker enzymes of muscles known to use glycolysis with different extent for ATP production are measured under identical assay conditions as enzymes of HUVEC. Additionally data from Board et al. (1990) for fibroblasts and tumorigenic cells are given [60]¹⁾.

to sP₃ (Fig. 2e, relative cell number calculated on the basis of quantified DNA amount) and Ki67 protein expression was reduced compared to sP₃ (Fig. 2f, reduction about 30%). 5 days after seeding in P₃ (cP₃) the cell number reached a steady state (Fig. 2e) and the Ki67 protein expression was even more reduced (Fig. 2f, reduction of about 50%). Hence, freshly isolated HUVEC did not show proliferative activity whereas subconfluent HUVEC (sP₃) showed substantial proliferation indicated by increased cell number and a marked nuclear Ki67 protein expression. Confluent cultures (cP₃) showed stable cell numbers and a considerable reduction of Ki67 protein expression and thus reduced proliferative activity.

Cultivation-dependent changes of enzyme activities in HUVEC

The activity ratios of marker enzymes of the different energy metabolic pathways are useful indicators for the favored metabolic pathways in tissues or cells [30]. Therefore, maximum catalytic activities of a number of key enzymes of important energy metabolic pathways were measured in freshly isolated HUVEC (P₀) and in HUVEC after about two weeks in culture (corresponding to 3 passages) harvested in a subconfluent (sP₃) and a confluent state (cP₃). All time points studied demonstrated the glycolytic enzymes (i.e. GAPDH, PFK) and LDH (indicating high capacities for lactate conversion) with the highest activities compared to the other metabolic pathways (Table 1). The maximum activity of GAPDH was 270, that of PFK was 17 and that of LDH reached 208 (unit: μmol product formed per min and g protein at 25°C). In comparison, maximum activities of mitochondrial metabolic pathways, i.e. ICDH (marker

enzyme for citric acid cycle) and HOADH (marker enzyme for fatty acid oxidation) were markedly lower (5.7 for ICDH and 1.4 for HOADH).

All enzyme activities were increased in isolated HUVEC within 2 weeks in culture but to a different extent. Compared to P₀, the enzyme activities in sP₃ were 3- to 5-fold higher with the exception of LDH (1.6-fold). The variances of enzyme activities between sP₃ (2 days after seeding) and cP₃ (5 days after seeding) indicated that the cytoplasmic enzyme activities (GAPDH, PFK, LDH, CK, and G-6P-DH) did not increase further, while the mitochondrial enzymes (ICDH and HOADH) did. Maximal activity of ICDH was completely NADP⁺-dependent in all three tested cell culture conditions (i.e. P₀, sP₃, cP₃). NAD⁺-dependent ICDH activity was not detectable in HUVEC in any of the tested states.

Comparison of HUVEC energy metabolic pattern with that of other tissues and cell types

We compared energy metabolic pattern of HUVEC with those of other tissues and cell types (Table 2). Since glycolysis appears to be important in HUVEC, we attributed most of these activities to PFK activity. Correspondingly, the ratio of HOADH/PFK of HUVEC was low (i.e. 0.12) similar to that of the white glycolytic frog muscle (i.e. 0.07), indicating the preference for glycolysis compared to fat oxidation. This ratio was 20-fold higher for mouse heart (HOADH/PFK-ratio in HUVEC was 0.12, in mouse heart 2.5) and 175-fold for the flight muscle of the mouth *M. sexta* (in HUVEC 0.12, in flight muscle 21.1). Correspondingly, the ratio of LDH to the marker enzyme of the citric acid cycle ICDH (NAD⁺ + NADP⁺-dependent activities) was low in this

flight muscle (i.e. 0.02) while HUVEC (i.e. 28), the white frog muscle (i.e. 130) and mouse heart (i.e. 30) have significant capacities for lactate production (e.g. white frog muscle) or lactate consumption (e.g. mouse heart). Very high LDH maximum activities are known to occur in tumorigenic or carcinoma cells resulting in high ratios of LDH/ICDH (Table 2; e.g. 111 in carcinoma cells, 135 in RCC-1t cells) and of LDH/PFK (e.g. 88.6 in epithelial carcinoma cells, 197.5 in RCC-1t cells).

G6PDH activity, related to the marker enzyme of glycolysis (PFK), was the highest in HUVEC (G6PDH/ PFK ratio of 0.68). The other proliferating cells (fibroblasts and tumor cells) also showed high G6PDH/ PFK ratios compared to muscles (e.g. MRC-5 fibroblasts: ratio of 0.4, rat heart cells RCC-1t: ratio of 0.2). A high capacity was also found for glutathione reductase (GSSG-red/ PFK, i.e. 0.32) in HUVEC which exceeded that of muscles several-fold (e.g. 0.02 in white muscle of frogs and 0.07 in red muscle of mice heart) and indicate a high defense potential against oxidative stress.

Discussion

EC undergo a variety of adaptations after isolation and cultivation *in vitro*. For example EC-typical markers such as vWF and CD31 were present in HUVEC *in situ* and their expression and cellular distribution did not change during 2 weeks of culture, whereas expression of other proteins such as CD34 (highly expressed in HUVEC *in situ*) decreased with cultivation time. The effect of reduced CD34 protein expression in culture was previously reported for human pulmonary EC [31]. The reason for this reduction in CD34 protein expression *in vitro* is unclear. CD34, formerly misleadingly used as an exclusive hematopoietic stem cell marker, is currently considered to be expressed in different progenitor cells of mesenchymal origin [32–34]. Therefore, reduction of CD34 expression could be a reflection of a specific differentiation status.

An additional example for cultivation-induced changes is the replication rate of human umbilical vein EC (HUVEC), which is low *in vivo* compared to that in culture, suggesting activation of a number of signaling pathways involved in cell proliferation. Cines *et al.* 1998, estimated the average replication rate of EC *in vivo* to about 0.1% per day and 10 to 20 % in culture [1]. The proliferation data of this study are completely in agreement with the estimation of Cines *et al.* in that freshly isolated HUVEC do not show substantial proliferative

activity (only a negligible proliferation marker/Ki67-positive population was detectable), whereas in subconfluent cultures of P₃ (sP₃) 20% of the cells were proliferating (Ki67 positive). Furthermore, we could show that subconfluent HUVEC cultures in P₃ increased cell numbers of about 2.4-fold within 2 days until reaching confluency (from sP₃ to cP₃). Cell proliferation decreased when cultures became confluent (cP₃) reaching almost a steady state in cell number between day 4 and 5 after seeding.

Against the background of divergent cultivation-induced reactions, we compared energy metabolic capacities of HUVEC directly after isolation and after 3 passages (i.e. two weeks in culture). The activity ratios of special marker enzymes are useful indicators for the favoured energy metabolic pathways in tissues or cells [30]. We found that the capacity for glycolysis with lactate production exceeded that of other pathways in HUVEC, independent of their proliferation state. This became obvious from the high maximum activities of LDH and GAPDH but also from PFK activity. The fact that the PFK activity is more than 10-fold lower than GAPDH is well known for many cell types and based on the fact that GAPDH catalyzes a near-equilibrium reaction *in vivo* while the regulatory enzyme PFK controls a non-equilibrium reaction and might limit the glycolytic flux [35]. According to the PFK activities of fresh and cultured HUVEC maximum glycolytic fluxes of about 2 to 8 μmol glucose/hour and mg cell protein at 37°C were calculated. This turnover is higher but in the same order of magnitude as the glycolytic flux in confluent rat coronary EC under aerobic conditions (0.7 μmol glucose/hour and mg cell protein at 37°C, calculated from data of Krützfeld *et al.* [21]). During hypoxia lactate production was not significantly increased (low Pasteur effect) which would be necessary to compensate aerobic ATP yield per glucose (38 ATP/glucose) by anaerobic lactate production (2 ATP/glucose). This also supports the hypothesis that in EC glycolysis with lactate production does not function as a compensation mechanism for reduced respiratory ATP-supply during hypoxia but is the main energy pathway even under aerobic conditions.

Our data on human EC support the theory that the energy metabolism of EC is characterized by glucose fermentation to lactate even under normoxic conditions (“aerobic glycolysis”) [24, 28]. The low ATP yield of “aerobic glycolysis” compared to respiration suggests important reasons to use this pathway. “Aerobic glycolysis” is known to occur in erythrocytes because these cells do not contain mitochondria [36]. It is also described for

sperm where the mitochondria are compartmentalized in the short midpiece of the flagellum and glycolysis may supply the mitochondrial-free region with ATP [37]. Warburg et al. observed “aerobic glycolysis” in various tumours (Warburg effect) and believed that an impairment of mitochondrial function caused “aerobic glycolysis” [38]. Overexpression of glycolytic enzymes appears to be typical for all tumour cell types so far tested but their capacity for oxidative phosphorylation seems to be tumour cell-specific [39, 40]. Studies on hepatocytes and several other non-malignant cell types have suggested that “aerobic glycolysis” is linked to cell proliferation rather than to malignancy [41]. A correlation was established between the amount of lactate produced and the cell doubling time [42]. Wang et al. investigated normal lymphocytes and proposed “aerobic glycolysis” not only associated with cellular proliferation, but more specifically temporally related to DNA synthesis [43]. Cultured rat thymocytes enter a proliferative cycle after stimulation by concanavalin A and interleukin-2, with maximal rates of DNA synthesis at 60 h. Compared with non-proliferating thymocytes, glucose metabolism of proliferating thymocytes was 53-fold increased [44]. Greiner et al. 1994, demonstrated that proliferating thymocytes cultured in glucose-containing medium induced their glycolytic enzymes 8-10-fold in the S phase of the cell cycle and the replacement of glucose by e.g. glutamine or glutamine and ribose prevents glycolytic enzyme induction and thymocyte proliferation [45]. The authors concluded that glucose was required for proliferation and for the glycolytic enzyme induction that mediated the transition from oxidative to glycolytic energy production during the cell cycle transition (G1/S) of rat thymocytes. In contrast, studies on chinese hamster fibroblasts totally blocked in their glycolytic pathway showed normal DNA synthesis indicating that growth-factor-stimulated glycolysis was not involved in the control of initiation of DNA synthesis and cell proliferation [46]. Our data showed that proliferation of EC in culture was accompanied by an increase of glycolytic enzymes, but even in a non-proliferative state glycolytic capacity was dominant, suggesting that “aerobic glycolysis” also occurred in non-proliferating EC and probably might be an adaptation to its function or the oxidative environment (see below). Whether the synthesis of glycolytic enzymes in EC is regulated on the transcriptional or translational level during proliferation is unclear. For *Saccharomyces cerevisiae* poor correlation between protein and mRNA levels indicated the importance of translational mechanisms and/or the control by protein degradation [47, 48]. The metabolic flux in EC is

certainly lower as the maximum activities of the enzymes because metabolic control by substrates, products and effectors of the flux generating enzymes like PFK for glycolysis limits the activities *in vivo* [49].

The presence of mitochondrial pathways indicated that ATP-supply of EC by “aerobic glycolysis” can be supported or partially replaced by respiration. In coronary EC, palmitate, lactate or glutamine are oxidized, particularly if no extracellular glucose is present [22]. Correspondingly, we found increasing activities of the mitochondrial enzymes HOADH and ICDH in HUVEC during proliferation, but their maximum activities are considerably lower than that of PFK. In contrast to the glycolytic enzymes and LDH, the activities of both mitochondrial enzymes still increased when HUVEC culture became confluent. This might be caused by the fact that the development of mitochondria requires more time during proliferation than the synthesis of glycolytic enzymes [50].

The CK activity in HUVEC was relatively low, particularly if compared with that in muscle (Tab. 2) using phosphocreatine for short term recovery of ATP during fast twitch contractions [51]. From pig coronary EC evidence is available that CK is located exclusively in the cytoplasm together with low phosphocreatine amounts [52] which are not permanently detectable by NMR [24]. Mitochondrial CK was not detected, thus arguing against a shuttle function of phosphocreatine/CK as proposed for various cell types particularly heart muscle or sperm [53, 54].

G6PDH is a key enzyme of the pentosephosphate pathway which delivers important substrates (NADPH and riboses) for anabolic metabolism. NADPH also serves for the maintenance of the cellular redox status and prevention of cell damage by oxidative stress. NADPH also contributes to endothelial NO-synthesis [55, 56]. Leopold et al. 2003, showed that G6PDH activity modulates EC migration, proliferation, and tube formation induced by VEGF (vascular endothelial cell growth factor) by regulating NO (nitric oxide) levels [57, 58]. A suggested mechanism is that G6PDH influences the activity via endothelial nitric oxide synthase (eNOS), which has an absolute requirement for NADPH as a cofactor. G6PDH may therefore serve as a regulatory determinant of the angiogenic phenotype [57]. The evidence and multifunctional role of G6PDH is represented by the comparatively high capacity of G6PDH activity not only in proliferating HUVEC but also in freshly isolated, non-proliferating HUVEC, indicating the importance of the pentose-phosphate pathway in EC (Table 2).

Another important regulating factor in mitochondria is NADPH. We showed that mitochondrial ICDH was completely dependent on NADP⁺. Furthermore, ICDH in HUVEC had a similar capacity as glutathione reductase, suggesting a function as a defensive mechanism against reactive oxygen species (ROS). ROS are byproducts particularly of respiration in mitochondria [59].

Summarizing, EC showed high capacities of “aerobic glycolysis” and of reactions protecting against oxidative stress not only during proliferation in culture but also in non-proliferating cells. Replacement of respiration by “aerobic glycolysis” can help to reduce the production of oxygen radicals. Therefore, we agree with Dobrina and Rossi that “aerobic glycolysis” of EC might be an adaptation to the oxidative environment [28]. During cell cultivation, which is associated with high proliferation

rates, the capacity of glycolysis increases, but those of other pathways as well. Hence this increase might only reflect the higher metabolic turnover during proliferation but not a shift, for example, from respiration to “aerobic glycolysis”.

Acknowledgements

This work was supported by the German Research Foundation (Deutsche Forschungsgemeinschaft, Priority program “Interface between material and biosystem” SPP1100, KI 601/1-1-4), the European Union and the Federal State of Mecklenburg-Vorpommern (V230-630-08-TMV-S/F-017). We wish to thank Anne Sartoris and Heike Stypa for their excellent technical assistance.

References

- Cines DB, Pollak ES, Buck CA, Loscalzo J, Zimmerman GA, McEver RP, Pober JS, Wick TM, Konkle BA, Schwartz BS, Barnathan ES, McCrae KR, Hug BA, Schmidt AM, Stern DM: Endothelial cells in physiology and in the pathophysiology of vascular disorders. *Blood* 1998;91:3527-3561.
- Busse R, Fleming I: Vascular endothelium and blood flow. *Handb Exp Pharmacol* 2006;43:78.
- Arnout J, Hoylaerts MF, Lijnen HR: Haemostasis. *Handb Exp Pharmacol* 2006;1:41.
- Peters K, Unger RE, Brunner J, Kirkpatrick CJ: Molecular basis of endothelial dysfunction in sepsis. *Cardiovasc Res* 2003;60:49-57.
- Risau W: Development and differentiation of endothelium. *Kidney Int Suppl* 1998;67:S3-6.
- Jaffe EA, Nachman RL, Becker CG, Minick CR: Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J Clin Invest* 1973;52:2745-2756.
- Grafe M, Auch-Schwelk W, Graf K, Terbeek D, Hertel H, Unkelbach M, Hildebrandt A, Fleck E: Isolation and characterization of macrovascular and microvascular endothelial cells from human hearts. *Am J Physiol* 1994;267:H2138-2148.
- Hewett PW, Murray JC: Human microvessel endothelial cells: Isolation, culture and characterization. *In Vitro Cell Dev Biol Anim* 1993;29A:823-830.
- Unger RE, Oltrogge JB, von Briesen H, Engelhardt B, Woelki U, Schlote W, Lorenz R, Bratzke H, Kirkpatrick CJ: Isolation and molecular characterization of brain microvascular endothelial cells from human brain tumors. *In Vitro Cell Dev Biol Anim* 2002;38:273-281.
- Kirkpatrick CJ, Krump-Konvalinkova V, Unger RE, Bittinger F, Otto M, Peters K: Tissue response and biomaterial integration: The efficacy of in vitro methods. *Biomol Eng* 2002;19:211-217.
- Kvietys PR, Granger DN: Endothelial cell monolayers as a tool for studying microvascular pathophysiology. *Am J Physiol* 1997;273:G1189-1199.
- Witting PK, Rayner BS, Wu BJ, Ellis NA, Stocker R: Hydrogen peroxide promotes endothelial dysfunction by stimulating multiple sources of superoxide anion radical production and decreasing nitric oxide bioavailability. *Cell Physiol Biochem* 2007;20:255-268.
- Thornhill MH, Haskard DO: IL-4 regulates endothelial cell activation by IL-1, tumor necrosis factor, or IFN- γ . *J Immunol* 1990;145:865-872.
- Unger RE, Krump-Konvalinkova V, Peters K, Kirkpatrick CJ: In vitro expression of the endothelial phenotype: Comparative study of primary isolated cells and cell lines, including the novel cell line hpmeec-st1.6r. *Microvasc Res* 2002;64:384-397.
- Peters K, Schmidt H, Kamp G, Unger RE, Hoffmann B, Stenzel F, Ziegler G, Jung M, Stumpf S, Kirkpatrick CJ: Software-supported quantification of angiogenesis in an in vitro culture system. Examples of applications in studies of basic research, biocompatibility and drug discovery; in Zubar RV (ed) *Trends in angiogenesis research*, Nova Science Publishers, 2005, pp 103-123.
- Peters K, Schmidt H, Unger RE, Otto M, Kamp G, Kirkpatrick CJ: Software-supported image quantification of angiogenesis in an in vitro culture system: Application to studies of biocompatibility. *Biomaterials* 2002;23:3413-3419.
- Fina L, Molgaard HV, Robertson D, Bradley NJ, Monaghan P, Delia D, Sutherland DR, Baker MA, Greaves MF: Expression of the cd34 gene in vascular endothelial cells. *Blood* 1990;75:2417-2426.
- Muller AM, Cronen C, Muller KM, Kirkpatrick CJ: Comparative analysis of the reactivity of human umbilical vein endothelial cells in organ and monolayer culture. *Pathobiology* 1999;67:99-107.
- Delia D, Lampugnani MG, Resnati M, Dejana E, Aiello A, Fontanella E, Soligo D, Pierotti MA, Greaves MF: Cd34 expression is regulated reciprocally with adhesion molecules in vascular endothelial cells in vitro. *Blood* 1993;81:1001-1008.
- Kanda T, Iwasaki T, Yamawaki M, Ikeda K: Isolation and culture of bovine endothelial cells of endoneurial origin. *J Neurosci Res* 1997;49:769-777.

- 21 Krutzfeldt A, Spahr R, Mertens S, Siegmund B, Piper HM: Metabolism of exogenous substrates by coronary endothelial cells in culture. *J Mol Cell Cardiol* 1990;22:1393-1404.
- 22 Spahr R, Krutzfeldt A, Mertens S, Siegmund B, Piper HM: Fatty acids are not an important fuel for coronary microvascular endothelial cells. *Mol Cell Biochem* 1989;88:59-64.
- 23 Loike JD, Cao L, Brett J, Ogawa S, Silverstein SC, Stern D: Hypoxia induces glucose transporter expression in endothelial cells. *Am J Physiol* 1992;263:C326-333.
- 24 Culic O, Gruwel ML, Schrader J: Energy turnover of vascular endothelial cells. *Am J Physiol* 1997;273:C205-213.
- 25 Decking UK, Alves C, Wallimann T, Wyss M, Schrader J: Functional aspects of creatine kinase isoenzymes in endothelial cells. *Am J Physiol Cell Physiol* 2001;281:C320-328.
- 26 Zhang Z, Apse K, Pang J, Stanton RC: High glucose inhibits glucose-6-phosphate dehydrogenase via camp in aortic endothelial cells. *J Biol Chem* 2000;275:40042-40047.
- 27 Gstraunthaler G, Seppi T, Pfaller W: Impact of culture conditions, culture media volumes, and glucose content on metabolic properties of renal epithelial cell cultures. Are renal cells in tissue culture hypoxic? *Cell Physiol Biochem* 1999;9:150-172.
- 28 Dobrina A, Rossi F: Metabolic properties of freshly isolated bovine endothelial cells. *Biochim Biophys Acta* 1983;762:295-301.
- 29 Bergmeyer J, Grasse M: Methods of enzymatic analysis. Weinheim, Verlag Chemie, 1983.
- 30 Pette D: Plan und Muster im zellulären Stoffwechsel. *Naturwissenschaften* 1965;52:597-616.
- 31 Muller AM, Hermanns MI, Skrzynski C, Nesslinger M, Muller KM, Kirkpatrick CJ: Expression of the endothelial markers pcam-1, vwf, and cd34 in vivo and in vitro. *Exp Mol Pathol* 2002;72:221-229.
- 32 Lama VN, Phan SH: The extrapulmonary origin of fibroblasts: Stem/progenitor cells and beyond. *Proc Am Thorac Soc* 2006;3:373-376.
- 33 Lin G, Garcia M, Ning H, Banie L, Gio YL, Lue TF, Lin CS: Defining stem and progenitor cells within adipose tissue. *Stem Cells Dev* 2008.
- 34 Seta N, Kuwana M: Human circulating monocytes as multipotential progenitors. *Keio J Med* 2007;56:41-47.
- 35 Newsholme EA, Leech AR: Biochemistry for the medical sciences. John Wiley & Sons Chichester, New York, 1983.
- 36 Murphy JR: Erythrocyte metabolism. II. Glucose metabolism and pathways. *J Lab Clin Med* 1960;55:286-302.
- 37 Feiden S, Stypa H, Wolfrum U, Wegener G, Kamp G: A novel pyruvate kinase (pk-s) from boar spermatozoa is localized at the fibrous sheath and the acrosome. *Reproduction* 2007;134:81-95.
- 38 Warburg O, Posener K, Negelein E: Ueber den stoffwechsel der tumoren. *Biochem Z* 1924;152:319-344.
- 39 Moreno-Sanchez R, Rodriguez-Enriquez S, Marin-Hernandez A, Saavedra E: Energy metabolism in tumor cells. *Febs J* 2007;274:1393-1418.
- 40 Walenta S, Schroeder T, Mueller-Klieser W: Lactate in solid malignant tumors: Potential basis of a metabolic classification in clinical oncology. *Curr Med Chem* 2004;11:2195-2204.
- 41 Markert CL: in McElroy WD, Glass B (eds): The chemical basis of development, John Hopkins University Press, Baltimore, 1958, pp 623-624.
- 42 Sweeney MJ, Ashmore J, Morris HP, Weber G: Comparative biochemistry hepatomas. Iv. Isotope studies of glucose and fructose metabolism in liver tumors of different growth rates. *Cancer Res* 1963;23:995-1002.
- 43 Wang T, Marquardt C, Foker J: Aerobic glycolysis during lymphocyte proliferation. *Nature* 1976;261:702-705.
- 44 Brand K: Glutamine and glucose metabolism during thymocyte proliferation. Pathways of glutamine and glutamate metabolism. *Biochem J* 1985;228:353-361.
- 45 Greiner EF, Guppy M, Brand K: Glucose is essential for proliferation and the glycolytic enzyme induction that provokes a transition to glycolytic energy production. *J Biol Chem* 1994;269:31484-31490.
- 46 Pouyssegur J, Franchi A, Salomon JC, Silvestre P: Isolation of a chinese hamster fibroblast mutant defective in hexose transport and aerobic glycolysis: Its use to dissect the malignant phenotype. *Proc Natl Acad Sci U S A* 1980;77:2698-2701.
- 47 Daran-Lapujade P, Rossell S, van Gulik WM, Luttik MA, de Groot MJ, Slijper M, Heck AJ, Daran JM, de Winder JH, Westerhoff HV, Pronk JT, Bakker BM: The fluxes through glycolytic enzymes in *saccharomyces cerevisiae* are predominantly regulated at posttranscriptional levels. *Proc Natl Acad Sci U S A* 2007;104:15753-15758.
- 48 Gygi SP, Rochon Y, Franza BR, Aebersold R: Correlation between protein and mRNA abundance in yeast. *Mol Cell Biol* 1999;19:1720-1730.
- 49 Newsholme EA, Crabtree B: Maximum catalytic activity of some key enzymes in provision of physiologically useful information about metabolic fluxes. *J Exp Zool* 1986;239:159-167.
- 50 Meyer R, Hackensellner AH: [on the behavior of the mitochondria of the endothelium of the aorta and pulmonary artery in tissue culture.]. *Z Zellforsch Mikrosk Anat* 1965;66:197-199.
- 51 Krause U, Wegener G: Control of glycolysis in vertebrate skeletal muscle during exercise. *Am J Physiol* 1996;270:R821-829.
- 52 Schrader J, Gruwel M, Decking U, Alves C: [why do endothelial cells require adenosine triphosphate?]. *Arzneimittelforschung* 1994;44:436-438.
- 53 Kalds P, Stolz M, Wyss M, Zanolla E, Rothen-Rutishauser B, Vorherr T, Wallimann T: Identification of two distinctly localized mitochondrial creatine kinase isoenzymes in spermatozoa. *J Cell Sci* 1996;109 (Pt 8):2079-2088.
- 54 Schlegel J, Zurbriggen B, Wegmann G, Wyss M, Eppenberger HM, Wallimann T: Native mitochondrial creatine kinase forms octameric structures. I. Isolation of two interconvertible mitochondrial creatine kinase forms, dimeric and octameric mitochondrial creatine kinase: Characterization, localization, and structure-function relationships. *J Biol Chem* 1988;263:16942-16953.
- 55 Hecker M, Mitchell JA, Swierkosz TA, Sessa WC, Vane JR: Inhibition by l-glutamine of the release of endothelium-derived relaxing factor from cultured endothelial cells. *Br J Pharmacol* 1990;101:237-239.
- 56 Hecker M, Sessa WC, Harris HJ, Anggard EE, Vane JR: The metabolism of l-arginine and its significance for the biosynthesis of endothelium-derived relaxing factor: Cultured endothelial cells recycle l-citrulline to l-arginine. *Proc Natl Acad Sci U S A* 1990;87:8612-8616.
- 57 Leopold JA, Walker J, Scribner AW, Voetsch B, Zhang YY, Loscalzo AJ, Stanton RC, Loscalzo J: Glucose-6-phosphate dehydrogenase modulates vascular endothelial growth factor-mediated angiogenesis. *J Biol Chem* 2003;278:32100-32106.
- 58 Leopold JA, Zhang YY, Scribner AW, Stanton RC, Loscalzo J: Glucose-6-phosphate dehydrogenase overexpression decreases endothelial cell oxidant stress and increases bioavailable nitric oxide. *Arterioscler Thromb Vasc Biol* 2003;23:411-417.
- 59 Lenaz G: The mitochondrial production of reactive oxygen species: Mechanisms and implications in human pathology. *IUBMB Life* 2001;52:159-164.
- 60 Board M, Humm S, Newsholme EA: Maximum activities of key enzymes of glycolysis, glutaminolysis, pentose phosphate pathway and tricarboxylic acid cycle in normal, neoplastic and suppressed cells. *Biochem J* 1990;265:503-509.